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Report

TRPV1 in GABAergic Interneurons Mediates Neuropathic Mechanical Allodynia and Disinhibition of the Nociceptive Circuitry in the Spinal Cord

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SUMMARY

Neuropathic pain and allodynia may arise from sensitization of central circuits. We report a mechanism of disinhibition-based central sensitization resulting from long-term depression (LTD) of GABAergic interneurons as a consequence of TRPV1 activation in the spinal cord. Intrathecal administration of TRPV1 agonists led to mechanical allodynia that was not dependent on peripheral TRPV1 neurons. TRPV1 was functionally expressed in GABAergic spinal interneurons and activation of spinal TRPV1 resulted in LTD of excitatory inputs and a reduction of inhibitory signaling to spinothalamic tract (STT) projection neurons. Mechanical hypersensitivity after peripheral nerve injury was attenuated in TRPV1$^{-/-}$ mice but not in mice lacking TRPV1-expressing peripheral neurons. Mechanical pain was reversed by a spinally applied TRPV1 antagonist while avoiding the hyperthermic side effect of systemic treatment. Our results demonstrate that spinal TRPV1 plays a critical role as a synaptic regulator and suggest the utility of central nervous system-specific TRPV1 antagonists for treating neuropathic pain.

INTRODUCTION

Pain hypersensitivity generated by peripheral injury can result from plastic changes in both the peripheral (Campbell and Meyer, 2006; Finnerup et al., 2007) and central nervous systems (CNSs) (Costigan et al., 2009; Coull et al., 2003; Ikeda et al., 2003). Mechanical allodynia, pain response to light touch, is the most common and challenging symptom found in pathological pain (Campbell and Meyer, 2006). The mechanisms underlying induction and maintenance of mechanical hypersensitivity are still uncertain (Costigan et al., 2009), but the dominant population of Nav1.8-expressing peripheral neurons that mediate acute mechanical and thermal pain are not required (Abrahamsen et al., 2008). The transmission of pain signals from primary afferent neurons to higher brain centers is controlled by a balance between excitatory and inhibitory signaling in the spinal cord dorsal horn (Kuner, 2010). A key area for pain processing is the substantia gelatinosa (SG) of the spinal dorsal horn and inhibitory SG interneurons have been proposed as a gate of pain transmission and other sensory modalities to higher brain centers (Melzack and Wall, 1965). It has been suggested that a reduction in tonic and phasic inhibitory control or ''disinhibition'' in the spinal dorsal horn is responsible for the amplification of pain messages that produces hyperalgesia and allodynia (Sivilotti and Woolf, 1994; Yaksh, 1989) following peripheral nerve injury (Basbaum et al., 2009; Moore et al., 2002). Thus, central rather than peripheral mechanisms appear to be responsible for the hyperexcitability of nociceptive signaling leading to neuropathic mechanical allodynia (Costigan et al., 2009; Coull et al., 2003; Torsney and MacDermott, 2006; Woolf et al., 1992).

TRPV1 antagonists have shown efficacy in animal models of both inflammatory and neuropathic pain (Patapoutian et al., 2009) but systemic administration of TRPV1 antagonists commonly results in hyperthermia caused by peripheral TRPV1 blockade (Steiner et al., 2007). Activation of spinal TRPV1 can generate central sensitization and mechanical allodynia (Patwardhan et al., 2009) and spinal administration of TRPV1 antagonists can attenuate mechanical allodynia induced by nerve injury (Patapoutian et al., 2009), but the cell types or circuits underlying these effects are unknown. Mechanical allodynia associated with TRPV1 activation is unlikely to depend on TRPV1-expressing primary sensory neurons as these are not necessary for the transduction of painful mechanical stimuli (Cavanaugh et al., 2009) and a mechanical pain phenotype is not observed in TRPV1$^{-/-}$ mice (Caterina et al., 2000). Thus, the mechanism of action for TRPV1 antagonism in neuropathic mechanical pain relief remains unknown. The expression of TRPV1 in spinal cord SG neurons has recently been suggested

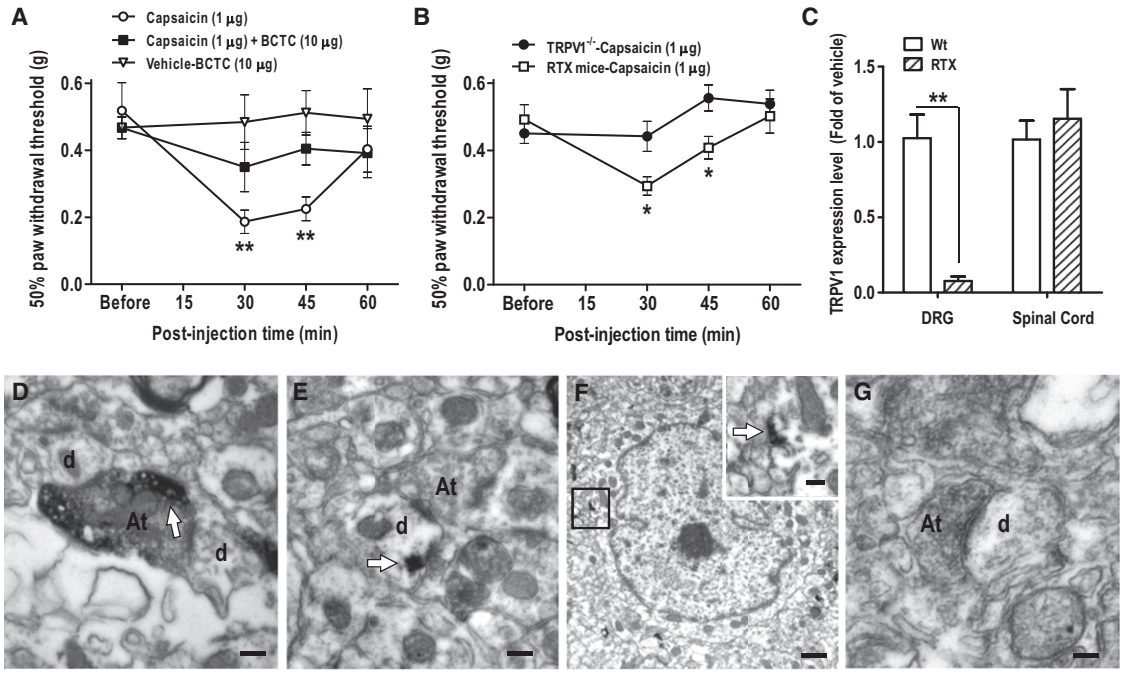

Figure 1. Spinal TRPV1 in Central Neurons Mediates Mechanical Allodynia

(A and B) Mechanical thresholds were measured after intrathecal administration of capsaicin. (A) Time course after injection of capsaicin (1 μg, n = 6), capsaicin (1 μg) with BCTC (10 μg, n = 6), and vehicle with BCTC alone (10 μg, n = 5) in naive mice. (B) Time course after injection of capsaicin (1 μg) in TRPV1$^{-/-}$ (n = 5) and RTX-treated mice (n = 6). One-way repeated-measures ANOVA of changes in mechanical threshold by capsaicin, *$p < 0.05$, **$p < 0.005$.

(C) Expression level of TRPV1 mRNA was markedly decreased in dorsal root ganglion but not in spinal cord (n = 3, unpaired t test; **$p < 0.001$) 7 days after intraperitoneal injection of RTX.

(D–G) Electron microscopic immunostaining for TRPV1 in the superficial lamina of the spinal dorsal horn in naive (D–F) and TRPV1$^{-/-}$ mice (G). (D) TRPV1 immunostaining is observed in an axon terminal containing spherical vesicles that is presynaptic to a dendrite, (E) in a dendrite that is postsynaptic to an axon terminal and (F) within somata (inset; higher magnification of boxed area). (G) TRPV1 immunostaining is completely abolished in the spinal dorsal horn of the TRPV1$^{-/-}$ mice. Arrow indicates TRPV1 immunoreaction product.

At, axon terminal; d, dendrite. Scale bar, 200 nm in (D)–(F) inset, and (G) and 1 μm in (F). All error bars represent SEM.

See also Figure S1 and Table S1.

(Ferrini et al., 2010). Therefore, we speculated that central TRPV1 may be involved in neuropathic mechanical pain. Here, we explored the role of spinal TRPV1 in the spinal cord nociceptive circuitry and further investigated its contribution to the enhancement of mechanical pain sensitivity after peripheral nerve injury.

RESULTS

Spinal TRPV1 Activation in SG Neurons Produces Mechanical Allodynia

We first examined the relative contribution of peripheral and central TRPV1 to the development of mechanical allodynia induced by the TRPV1 agonist capsaicin. Consistent with a recent report (Patwardhan et al., 2009), intrathecal administration of capsaicin in mice decreased the paw withdrawal mechanical threshold (Figure 1A). This effect was prevented by coadministration of the TRPV1-selective antagonist N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carbox-amide (BCTC, Figure 1A). Capsaicin had no effect on mechanical thresholds in TRPV1$^{-/-}$ mice (Figure 1B). Intrathecal capsaicin could act on either postsynaptic TRPV1 expressed in spinal cord neurons or on presynaptic TRPV1 expressed at the primary afferent terminals of sensory neurons. To distinguish between these possibilities, we generated mice in which TRPV1-expressing peripheral neurons are ablated by intraperitoneal injection of the ultrapotent TRPV1 agonist resiniferatoxin (RTX). RTX treatment eliminated TRPV1 mRNA in dorsal root ganglia without altering mRNA levels in the spinal cord (Figure 1C). RTX treatment appeared to be effective in completely ablating peripheral neurons expressing TRPV1, including their central terminals, because there was complete loss of TRPV1 immunoreactivity in DRG neurons (Figures S1A and S1B available online), nearly complete (98%) loss of capsaicin-induced calcium increases in DRG cell bodies (Figure S1D), and complete loss of capsaicin response of presynaptic terminals (Figure S1C). However, in contrast to TRPV1$^{-/-}$ mice, intrathecal capsaicin injection was still able to effectively induce mechanical hypersensitivity in RTX-treated mice (Figure 1B), suggesting a site of action on central neurons. High-resolution electron microscopic analysis of the lumbar spinal cord revealed TRPV1 was localized not only to presynaptic terminals, as expected (Figure 1D), but also

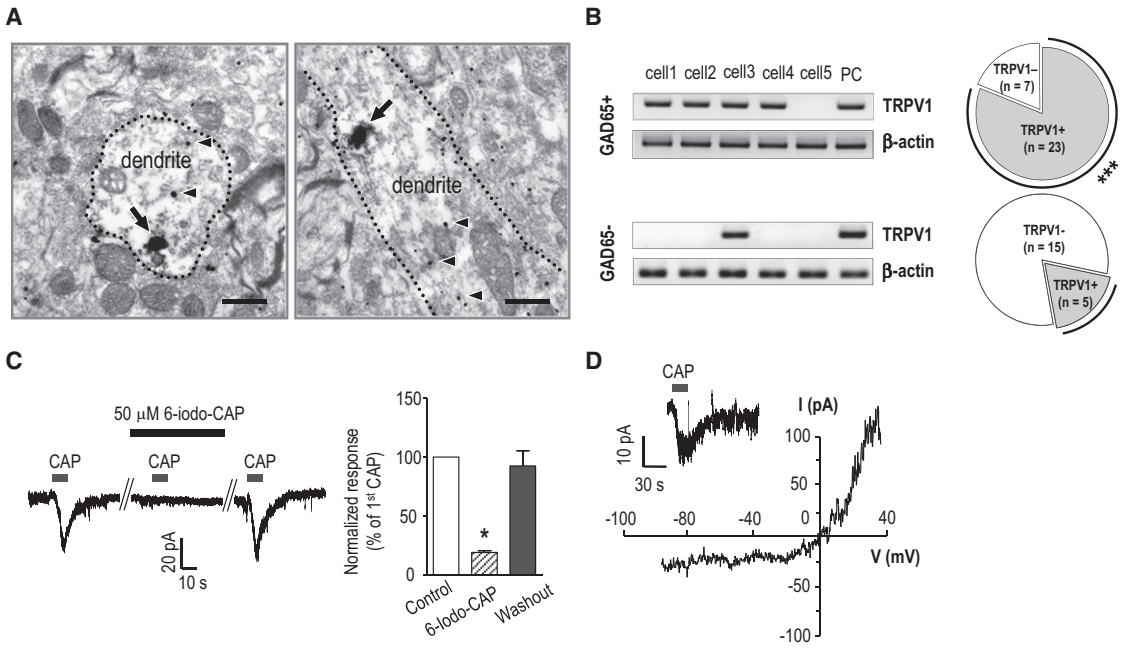

Figure 2. TRPV1 Is Functionally Expressed by GAD-Positive SG Neurons

(A) Electron micrograph of immunoperoxidase staining for TRPV1 combined with immunogold labeling for glutamic acid decarboxylase (GAD) in spinal dorsal horn of mice. TRPV1 (arrow) and GAD (arrow head) were detected in the same dendrites. Scale bar, 500 nm.

(B) Single-cell RT-PCR revealed that TRPV1 mRNA was expressed predominantly in a population of GAD65-EGFP positive SG neurons (n = 23/30) but in also in a small population of GAD65-EGFP negative SG neurons (n = 5/20, ***p = 0.0005, Fisher's exact test).

(C and D) Functional expression of TRPV1 in GAD65-EGFP positive SG neurons. (C) Capsaicin (CAP, 2 μM)-induced currents were blocked by 50 μM 6-iodo-nordihydrocapsaicin (6-iodo-CAP, n = 6, *p = 3.61e-8). (D) I–V relationship (−90 to +40 mV) obtained from capsaicin-induced currents.

All error bars represent SEM.

See also Figure S2 and Table S2.

to postsynaptic dendrites in the dorsal horn (Figure 1E), and postsynaptic cell soma (Figure 1F). No TRPV1 immunoreactivity was observed in the dorsal horn of TRPV1$^{-/-}$ mice (Figure 1G).

TRPV1 Is Functionally Expressed by GABAergic SG Neurons

We next sought to identify the population of postsynaptic spinal cord neurons that functionally express TRPV1. SG neurons of the spinal dorsal horn are a heterogenous population of interneurons (Maxwell et al., 2007; Todd and McKenzie, 1989) that receive direct inputs from primary afferent fibers (Yasaka et al., 2007). TRPV1 immunoreactivity was colocalized in postsynaptic dendrites with the GABA synthesizing enzyme glutamic acid decarboxylase 65 (GAD65) (Figure 2A). TRPV1 mRNA was detected in 76.7% of GAD65-enhanced green fluorescent protein (EGFP) positive SG neurons by single-cell RT-PCR (Figure 2B, upper). In contrast, the occurrence of TRPV1 mRNA in GAD65-EGFP negative SG neurons was lower (25%, Figure 2B, lower). To test for functional expression of TRPV1, we applied capsaicin (CAP) to spinal cord slices while recording from SG neurons. In addition to eliciting spontaneous excitatory postsynaptic currents (EPSCs), as expected from activation of presynaptic TRPV1 (Figure S2A), capsaicin also elicited clear whole-cell currents in SG neurons (Figure S2B) that persisted in the presence of a cocktail of neurotransmission blockers including 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), amino-5-phosphonovaleric acid (AP5), picrotoxin, strychnine, and tetrodotoxin with high $MgCl_2$ and a calcium chelator (see Experimental Procedures). Whole-cell capsaicin-induced currents (16.5 ± 2.5 pA) were recorded in identified GAD65-positive SG neurons; these currents were blocked by the TRPV1 antagonist 6-iodo-nordihydrocapsaicin (6-iodo-capsaicin, 18.70% ± 1.47%, Figure 2C) and showed outward rectification with a reversal potential of ~0 mV characteristic of TRPV1-mediated currents (Caterina et al., 1997; Figure 2D and Figure S2C). A high proportion of these functionally TRPV1-positive, GAD65-positive SG neurons displayed a long-lasting tonic- or phasic-firing pattern (Figure S2D) characteristic of inhibitory spinal cord interneurons (Cui et al., 2011). These results show that TRPV1 is functionally expressed in a substantial subpopulation of GABAergic SG neurons.

Postsynaptic Spinal TRPV1 Mediates LTD via AMPA Internalization in GABAergic SG Neurons and Results in Depression of Inhibitory Input to Projection Neurons

We next examined the role of postsynaptic spinal TRPV1 in the spinal cord synaptic circuitry involving GAD65-positive SG neurons. Application of capsaicin induced a long-lasting depression of EPSCs evoked in SG neurons by electrical stimulation of the dorsal root entry zone (DREZ). This effect of capsaicin was

abolished in slices prepared from TRPV1$^{-/-}$ mice and also when intracellular 6-iodo-capsaicin was introduced by the patch pipette (Figure 3A). Consistent with a postsynaptic action of capsaicin in LTD, the inclusion of 6-iodo-capsaicin in the patch pipette did not inhibit spontaneous EPSCs induced by presynaptic-TRPV1 activation (Figure S3A). The capsaicin-induced LTD persisted in RTX-treated mice (Figure 3B) and capsaicin did not affect the paired-pulse ratio (Figure 3C), suggesting that the LTD is independent of TRPV1-expressing afferents and is not mediated by changes in presynaptic neurotransmitter release. Capsaicin-induced LTD was not observed when intracellular calcium was buffered by 1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA) in the recording pipette (Figure 3B) confirming that elevation of postsynaptic of calcium is required for synaptic depression by capsaicin. The capsaicin-induced LTD of EPSC was not dependent on the activity of NMDA receptors, group I and II metabotropic glutamate receptors (mGluR), or the substance P receptor neurokinin 1 as the effect was not blocked by application of the antagonists AP5 (50 μM), Hexyl-HIBO (HIBO, Group I mGluR antagonist, 200 μM), LY341495 (Group II mGluR antagonist, 100 μM) (Figure 3B), (RS)-α-methyl-4-carboxyphenylglycine (MCPG, nonselective group I/group II mGluR antagonist, 500 μM) and L-703,606 (10 μM) (Figure S3B), respectively. Thus, we tested the involvement of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors as a likely candidate mediating TRPV1-dependent synaptic inhibition. We observed that whole-cell currents elicited by focal application of AMPA were reduced after capsaicin application (68.08% ± 3.39%, Figure 3D); the reduction in AMPA current was not observed in the presence of 6-iodo-capsaicin (Figures S3C and S3D). This finding was consistent with a postsynaptic locus and suggested altered membrane expression of AMPA receptors. Indeed, following capsaicin application to spinal cord slices we observed a reduction in membrane expression of AMPA receptor subunit GluR2 protein (60.4% ± 9.8%), the main AMPA subunit in the SG (Polgár et al., 2008; Figure 3E).

To examine the functional consequences of capsaicin-induced LTD in GABAergic SG interneurons, we retrogradely labeled spinothalamic tract (STT) projection neurons by injection of 1,1′,dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) into the ventroposterolateral (VPL) subnucleus of the thalamus (Figure 3F). Labeled neurons were located in the deep lamina of the spinal dorsal horn and showed inhibitory postsynaptic currents (IPSCs) in response to DREZ stimulation (Figure 3F and Figure S4) that were blocked by CNQX (10 μM) and AP5 (50 μM), confirming their polysynaptic nature. The amplitude of DREZ-evoked IPSCs in STT neurons from wild-type (Wt) and RTX-treated mice was decreased after capsaicin application, and depression of IPSCs (Wt, 56% ± 11%; RTX-treated mice, 65% ± 9%) lasted for at least 15 min (Figure 3F). The reduction in IPSC amplitude was not the result of a direct action of capsaicin on STT neurons as TRPV1 mRNA was not detected in STT neurons by single-cell RT-PCR (Figure 3F). Together, these data suggest that activation of TRPV1 leads to depression of excitatory input to GABAergic SG interneurons by a postsynaptic mechanism involving intracellular calcium-dependent GluR2 internalization, thus resulting in reduced inhibitory input to STT neurons (Figure 3G).

Postsynaptic Spinal TRPV1 Is Involved in the Maintenance of Chronic Mechanical Allodynia after Nerve Injury

To determine whether activation of spinal TRPV1 plays a role in the development of neuropathic pain, we measured mechanical sensitivity in a chronic constriction injury (CCI) model. Accumulating mechanical hypersensitivity up to 28 days after CCI was attenuated by ~41% in TRPV1$^{-/-}$ mice (Figures 4A and 4C) but not in RTX-treated mice (Figure 4B and 4C). Furthermore, spinal TRPV1 inhibition by intrathecal administration of BCTC dose-dependently alleviated chronic mechanical pain in RTX-treated mice following CCI (Figures 4D and 4E). By restricting TRPV1 blockade to the spinal cord central nervous system (CNS) using intrathecal injection, we were able to avoid the induction of hyperthermia that occurred with systemic (intravenous) administration of BCTC (Figure 4F).

DISCUSSION

We have shown that activation of postsynaptic spinal TRPV1 leads to decreased functional AMPA receptor expression in GABAergic SG interneurons and thus reduced excitation of a key population of inhibitory interneurons. Our observation of reduced inhibitory synaptic signaling to STT neurons of the deep lamina suggests a mechanism of disinhibition of spinal cord projection neurons that are critical for the relay of nociceptive signals to higher brain centers. Using the sciatic nerve CCI model in TRPV1$^{-/-}$ mice we uncover a substantial role of TRPV1 in neuropathic mechanical pain. We observed ~41% reversal of CCI-induced mechanical allodynia by spinal application of the TRPV1 antagonist BCTC in RTX-treated mice (Figure 4D and 4E) revealing that endogenous activation of spinal TRPV1, possibly by GPCRs (Kim et al., 2009) or arachidonic acid (AA) metabolites (Gibson et al., 2008) such as 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (Figure S5) contributes to the maintenance of chronic mechanical allodynia after neuropathic nerve injury. Our observations also correlate with findings showing that TRPV1 antagonists with greater CNS penetration are more potent for reducing mechanical allodynia (Cui et al., 2006; Patapoutian et al., 2009). Finally, we have shown that by targeting spinally mediated chronic pain we can avoid the side effects of peripheral TRPV1 blockade on temperature homeostasis (Steiner et al., 2007).

Our results help to clarify prior controversy surrounding the role of TRPV1 by explaining how it is that TRPV1 antagonists can reduce neuropathic mechanical pain (Cui et al., 2006; Patapoutian et al., 2009) even though TRPV1-expressing primary sensory neurons do not convey physiological mechanical pain (Cavanaugh et al., 2009). By using RTX to ablate TRPV1-expressing primary afferents, we were able to functionally isolate the contribution of postsynaptic TRPV1; however, further study into spinal TRPV1-mediated plasticity may require conditional TRPV1 knockout in DRG neurons. A recent study using a TRPV1 reporter mouse showed that there are very few cells in the CNS that express TRPV1 (Cavanaugh et al., 2011); our results using both immuno EM and electrophysiology show that a subpopulation of interneurons in the SG are among these. The TRPV1-mediated currents in these SG neurons were small

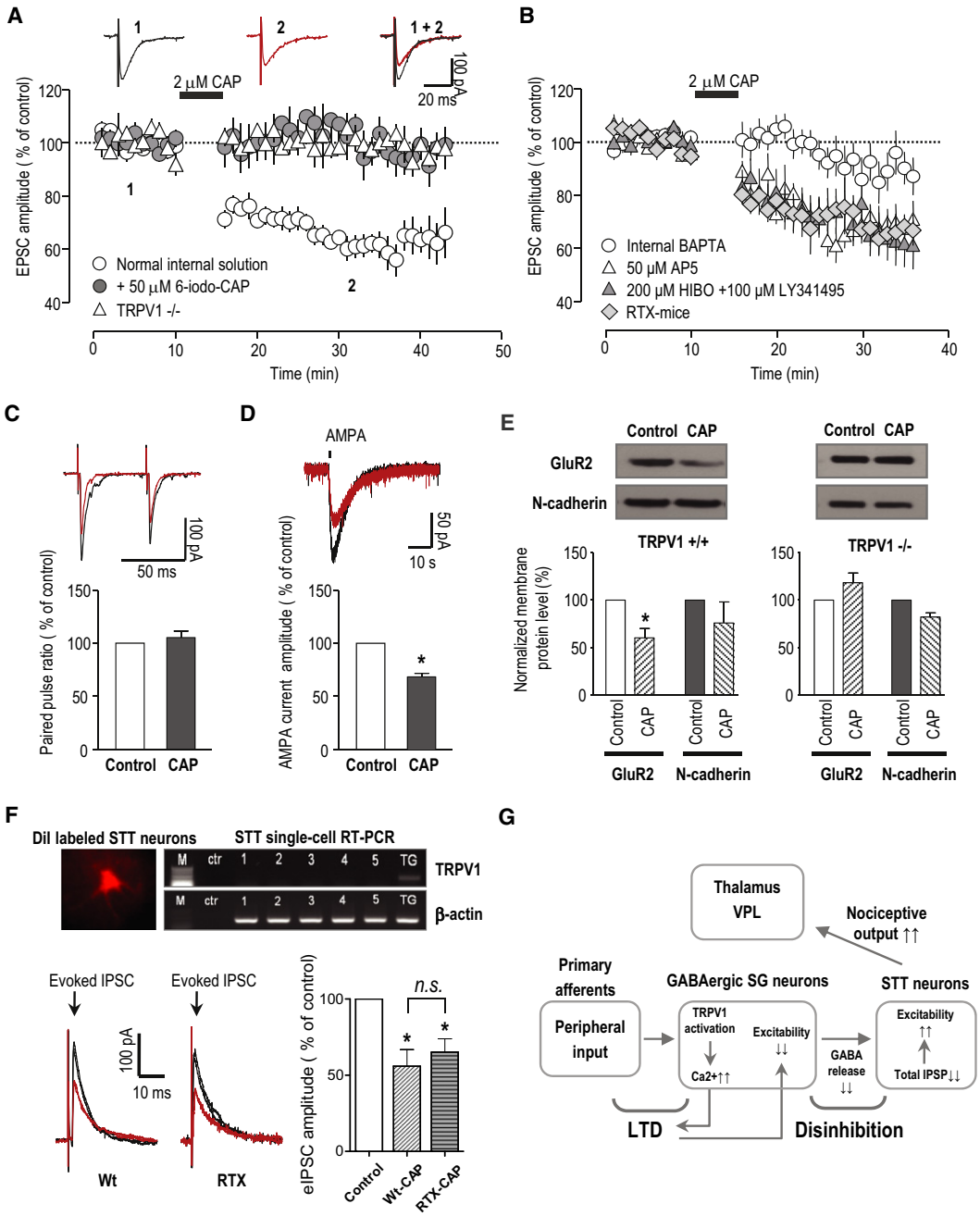

Figure 3. Capsaicin-Induced LTD via Reduction of Membrane GluR2 in GAD-Positive SG Neurons Results in Depression of Inhibitory Input to STT Neurons in Spinal Cord

(A) CAP (2 μM for 5 min) induced LTD of eEPSCs (V_h = −70 mV, n = 9) in GAD65-EGFP positive SG neurons that was blocked by intra-pipette 6-iodo-CAP (50 μM, n = 5) and was absent in TRPV1$^{-/-}$ mice (n = 5).

(B) CAP-induced LTD was blocked by internal administration of calcium chelator, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA, 10 mM, n = 6), but not by amino-5-phosphonovaleric acid (AP5, NMDA-R blocker, 50 μM, n = 8) or Hexyl-HIBO (HIBO, Group I mGluR antagonist, 200 μM) with LY341495 (Group II mGluR antagonist, 100 μM) (n = 7). Administration of CAP consistently induced LTD in RTX-treated mice (n = 6).

(C) Paired pulse ratio was obtained by a pair of stimuli given at 50 ms intervals (n = 7).

(D) AMPA-induced currents were elicited by 100 μM AMPA puffing (20–200 ms, 3 min interval repeated puffing) at −70 mV holding potential. Bath application of capsaicin (2 μM, 5 min) decreased AMPA-induced currents (n = 6, *p = 2.27e-4).

(E) GluR2 receptors in membrane fraction was reduced by CAP (5 μM for 10 min and washout for 30 min) in lumbar spinal cord of wild-type mice, but not in TRPV1$^{-/-}$ mice (n = 3, for each group, *p = 0.016).

(F) Single-cell RT-PCR revealed no mRNA expression of TRPV1 in spinothalamic tract (STT) neurons. In STT neurons from both wild-type (Wt) and RTX-treated mice, evoked IPSCs at 0 mV following stimulation of dorsal root entry zone (DREZ) were reduced by CAP (2 μM for 5 min and washout for 10 min, Wt;

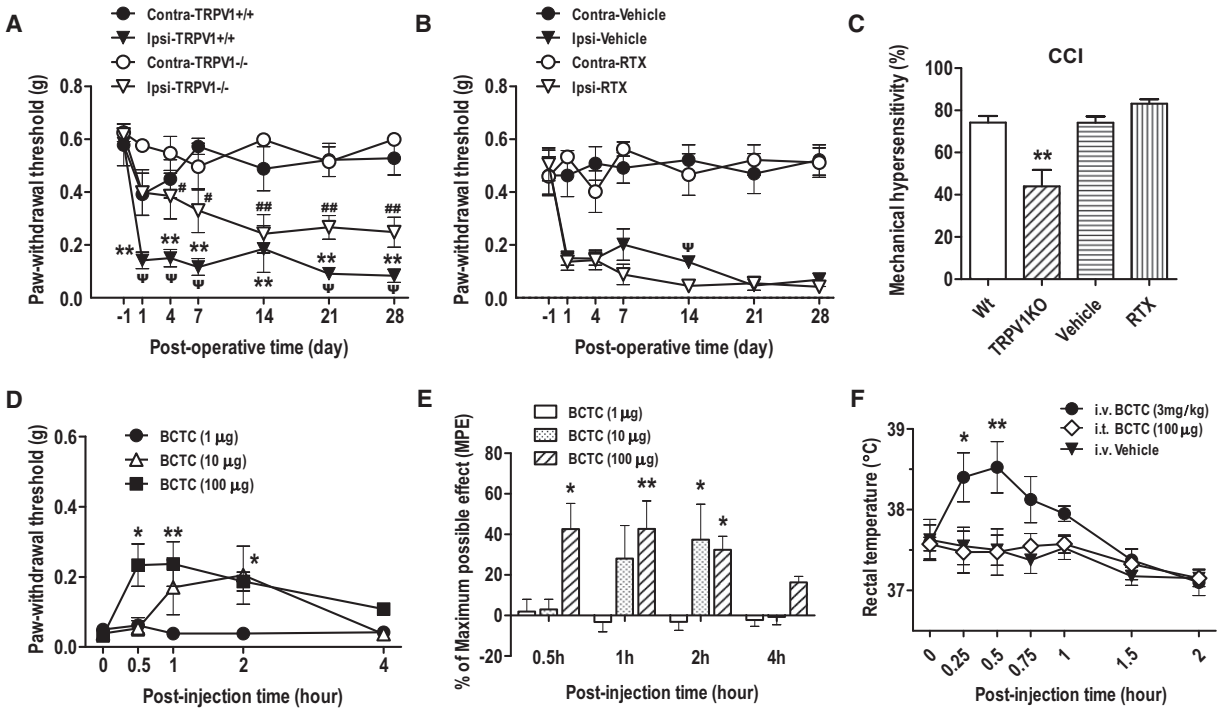

Figure 4. Chronic Mechanical Allodynia by Nerve Injury Is Alleviated by Blockade of Postsynaptic TRPV1 in Spinal Cord

(A and B) Changes in the mechanical thresholds after sciatic nerve chronic constriction injury (CCI) were measured in TRPV1$^{+/+}$, TRPV1$^{-/-}$, vehicle-treated and RTX-treated mice (n = 6 for each group). One-way repeated-measures ANOVA followed by Bonferroni's test; **p < 0.001 (naive mice), #p < 0.05, ##p < 0.001 (TRPV1$^{-/-}$ mice versus Presurgical value (−1 day), t test; Ψ p < 0.05 (TRPV1$^{+/+}$ versus TRPV1$^{-/-}$ or vehicle-treated versus RTX-treated).

(C) Mechanical hypersensitivity was calculated as the percentage difference in the mechanical thresholds of ipsilateral and contralateral hind paws accumulated from each time point up to 28 days after CCI (n = 6 for each group). One-way ANOVA followed by Bonferroni's test; *p = 0.0051 (Wt versus TRPV1$^{-/-}$).

(D) Intrathecal injection of BCTC in RTX-treated mice reversed chronic mechanical hypersensitivity at 28 days after CCI in a dose-dependent manner (n = 6 for each group).

(E) The data were normalized and displayed as the maximum possible effect (MPE). Two-way ANOVA, Bonferroni's test; *p < 0.05, **p < 0.01.

(F) Rectal body temperature measured after intravenous (i.v.) injection of BCTC (3 mg/kg) or vehicle only (50% DMSO in saline) compared with high dose intrathecal (i.t.) injection of BCTC (100 μg). (n = 4 mice per group).

Two-way ANOVA, Bonferroni's test; *p < 0.05, **p < 0.01. All error bars represent SEM.

See also Figure S5.

(~17 pA on average), corresponding to activation of only a few dozen TRPV1 channels. Nevertheless, we find that this sparse expression of postsynaptic TRPV1 channels in a key population of neurons has major functional consequences, playing a critical role in mediating mechanical allodynia.

Together with TRPV1-mediated synaptic plasticity recently demonstrated in hippocampus (Gibson et al., 2008), dentate gyrus (Chávez et al., 2010), and nucleus accumbens (Grueter et al., 2010), this work provides further evidence for the functional significance and physiological implications of TRPV1 in the CNS. In particular, our results show that TRPV1 expression in a key population of spinal cord neurons underlies a critical role as modulator of pain transmission in spinal circuits distinct from its well-known role as a molecular transducer of pain in primary sensory neurons.

EXPERIMENTAL PROCEDURES

Detailed protocols are listed in Supplemental Experimental Procedures.

Animals

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the School of Dentistry, Seoul National University. Animal treatments were performed according to the guidelines of the International Association for the Study of Pain. Adult C57BL/6J (wild-type) male mice, heterozygous GAD65-EGFP mice, and TRPV1$^{-/-}$ mice of C57BL/6J background were used.

n = 6, RTX; n = 7, One-way ANOVA, Bonferroni's test; *p < 0.05 (Control versus Wt-CAP or RTX-CAP group), *n.s.* (Wt-CAP versus RTX-CAP group). n.a., nonsignificant.

(G) Schematic representation of TRPV1 activation in GABAergic SG neurons and hypothesized sequence of events for the genesis of pain hypersensitivity through disinhibition of nociceptive circuitry in the spinal cord.

All error bars represent SEM.

See also Figures S3 and S4 and Table S2.

Behavior Studies

To assess mechanical sensitivities, the withdrawal threshold of the hindpaw was measured using a series of von Frey filaments (Stoelting, Wood Dale, IL). All behavioral testing was performed by an investigator who was blind to the treatment group and genetic background of the mice.

Intrathecal Injection

Drugs or vehicle (5 μl) was injected at the level of the lumbar enlargement using a 25 μl Hamilton syringe fitted with a 31 gauge needle.

RTX Ablation of Peripheral TRPV1-Expressing Neurons

Three- to four-week-old mice were intraperitoneally injected with RTX dissolved in a mixture of 10% Tween-80 and 10% ethanol in normal saline or vehicle alone under isoflurane anesthesia as a single bolus in two injections of 50 μg/kg and 150 μg/kg on days 1 and 2, consecutively. RTX-treated mice were used in experiments at least 7 days after final RTX injection.

Real-Time RT-PCR

Real-time PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems). All ΔCt values were normalized to GAPDH. The PCR primer sequences used in this study are listed in Table S1A.

Visualized Whole-Cell Patch Clamp Recordings

Transverse slices (300 μm) were prepared from C57BL/6J or GAD65-EGFP mice (4–6 weeks old). Whole-cell patch clamp recordings of spinal cord SG and STT neurons were performed at room temperature (25°C ± 1°C). To prevent spontaneous synaptic activity, a cocktail of neurotransmission inhibitors were added (in μM): 10 CNQX; 50 D-AP5, 10 picrotoxin, 2 strychnine, 0.5 tetrodotoxin. For the composition of all internal and modified aCSF solutions see Supplemental Experimental Procedures.

Synaptically Evoked Current Recordings

To record EPSCs, SG neurons were held at −70 mV. Electrical stimuli (0.01 ms, 0.066 Hz) were delivered through a bipolar, Teflon-coated tungsten electrode, which was placed in DREZ of spinal cord and monosynaptic EPSCs were identified on the basis of the absence of conduction failure of evoked EPSCs. To record evoked inhibitory postsynaptic currents (eIPSCs) from STT neurons, the DiI-labeled neurons were held at 0 mV.

Single-Cell RT-PCR

Under fluorescence microscopy, GAD65-EGFP SG neurons and DiI-labeled STT neurons were verified in spinal cord slices. Identified cells were collected into a patch pipette with a tip diameter of about 20 μm and gently put into a reaction tube containing reverse transcription reagents. All PCR amplifications were performed with nested primers (Table S1B).

Western Blotting Analysis

Spinal cord slices (700 μm) were incubated in 95% O_2/5% CO_2 saturated recording aCSF with 5 μM capsaicin for 10 min at 32°C followed by washed out for 30 min. Spinal cord slices were homogenized and centrifuged and protein concentration was determined with BCA assay kit (Pierce). Equal amounts of proteins were separated by SDS-PAGE electrophoresis and transferred onto PVDF membrane. After blocking, the membrane was probed with primary antibodies overnight followed by horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Blots were developed by chemiluminescence reagent (West-zol, Intron) exposure to photographic film and quantified. Independent experiments were conducted at least three times.

Application of Retrograde Tracer into VPL Region of Thalamus

Under pentobarbital sodium (40 mg/kg) anesthesia, DiI (Molecular Probes; 1 μl, 25 mg/0.5 ml in ethanol) was injected into the VPL region of the thalamus (Bregma: −1.2 ± 0.2 mm, midline: 1.9 ± 0.2 mm, depth: 3.2 ± 0.2 mm) using a glass micropipette (20 μm tip diameter) which was guided to the target area using a stereotaxic apparatus (Narishige, Tokyo, Japan).

Chronic Constriction Injury Model

Two silk sutures (7-0; Ailee, Busan, Korea) were tied loosely around the full circumference of the sciatic nerve 2–3 mm apart and secured with a reef knot; intraneural blood flow was not impeded. For reversal of chronic mechanical allodynia, BCTC was intrathecally injected at 28 days after CCI surgery.

Rectal Temperature Recording

Rectal temperature was measured by insertion of a flexible bead probe with a digital thermometer (TC-324B, Warner Instrument Corp., Hamden, CT).

Drugs

All drugs were made as stock solutions and keep at −20°C and diluted as final concentration (1:1,000–5,000).

Statistical Analyses

We expressed data as mean ± SEM, unless otherwise indicated. Significances in 50% paw withdrawal thresholds in comparison with preinjection or preinjury levels were calculated by one-way repeated-measure ANOVA followed by Bonferroni's post-test and Student's unpaired t test.

Detailed methodology can be found in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2012.02.039.

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